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Salivary IgA: A biomarker for resistance to *Teladorsagia circumcincta* and a new estimated breeding value

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1 Salivary IgA: a biomarker for resistance to *Teladorsagia*
2 *circumcincta* and a new Estimated Breeding Value

3

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35

36 Abstract

37 *Teladorsagia circumcincta* is the dominant nematode of
38 sheep in cool, temperate climates. Faecal nematode egg
39 counts (FEC) are widely used to identify the intensity of
40 infection and as a measure of host resistance to nematodes.
41 However due to density-dependent effects on worm
42 fecundity the relationship between FEC and worm burden
43 is not linear. In addition collecting FEC data is challenging
44 on a practical level and there is a need for more reliable
45 markers of resistance. There are two major known
46 mechanisms of immunity to *T. circumcincta*: IgE against
47 third stage larvae (L3), which inhibits larval establishment
48 and IgA against fourth stage larvae (L4), which inhibits
49 parasite growth. We measured salivary IgA responses in
50 over 5000 animals against L3 antigen by Enzyme Linked
51 Immunosorbent Assay (ELISA). Antigen-specific IgA levels
52 were negatively correlated with FEC ($r=-0.26$, $SE=0.02$) and
53 were heritable ($h^2 = 0.16$, $SE=0.04$) indicating that they can
54 be used to identify resistant animals suitable for inclusion
55 in selective breeding programs. Ecological theory predicts
56 that a trade-off between immunity and host-growth will
57 exist due to competing energetic needs. Antigen-specific IgA

58 responses were not negatively correlated with muscle
59 deposition such that the expected trade-off between growth
60 and immunity was not apparent. Our analyses indicate that
61 selection for antigen-specific IgA is possible without
62 impacting on the production traits for the Lleyn breed.

63

64 1. Introduction

65 Infection with gastro-intestinal strongyles such as
66 *Teladorsagia circumcincta* presents a significant problem to
67 the sheep breeding industry, impacting both the welfare
68 and productivity of animals. Anthelmintics are commonly
69 used to control helminth infection but reliance on this
70 method alone is ill-considered in light of growing
71 anthelmintic resistance (Traversa and von Samson-
72 Himmelstjerna, 2016). An integrated approach to ensure
73 sustainable control strategies for the future is the main
74 objective of research in this area and requires improved
75 phenotypic and genetic markers of resistance and resilience
76 to inform selective breeding programs (Jackson et al.,
77 2009).

78

79 Whilst both resistance and resilience contribute to control
80 of helminth infection it may be more preferable when
81 considering productivity to select for resilient sheep that
82 can better tolerate the infection and grow in spite of worm

83 burden. Previous research has demonstrated that there are
84 two main mechanisms of immunity to *T. circumcincta*
85 infection. Immunoglobulin E (IgE) against third stage larvae
86 (L3), which inhibits larval establishment and
87 Immunoglobulin A (IgA) against fourth stage larvae (L4),
88 which inhibits parasite growth (Lee et al., 2011; Martinez-
89 Valladares et al., 2005; Murphy et al., 2010; Strain et al.,
90 2002). The IgE response, although protective is associated
91 with pathology that leads to a relative protein deficiency in
92 the animal (Stear et al., 2003). Thus resistance to the
93 infection is costly both in terms of welfare and productivity.
94 The IgA response impairs the growth of the worms
95 resulting in shorter and crucially less fecund worms that
96 contribute less to pasture contamination (Stear et al., 1995;
97 Strain et al., 2002). IgA without the pathology and negative
98 impact on animal growth associated with IgE has potential
99 as a marker of resilience.

100

101 Traditionally low faecal nematode-egg count (FEC) is used
102 as a marker of resistance in selective breeding programmes.
103 However, mathematical modelling indicates that IgA has
104 advantages over FEC as a selection tool; after 7 generations
105 of selection based on plasma IgA a drop in FEC of 85% was
106 achieved whereas selection on FEC alone gave only a 50%
107 reduction (Prada Jimenez de Cisneros et al., 2014). There

108 are also other disadvantages of using FEC as a measure of
109 infection intensity; worm density-dependent effects on egg
110 production mean that high worm burdens do not always
111 tally with high FEC (Bishop and Stear, 2000; Romeo et al.,
112 2014; Smith et al., 1987), sampling per rectum is invasive
113 and often samples can't be obtained. Measuring IgA in
114 plasma is also invasive and requires specialist training.

115

116 Salivary IgA to a carbohydrate larval antigen (CARLA) has
117 been used successfully to measure immunity to
118 gastrointestinal nematodes (Shaw et al., 2012). Here we
119 have developed an Enzyme Linked Immunosorbent Assay
120 (ELISA) to measure *T. circumcincta*-specific IgA in saliva.
121 The test measures the IgA response to L3 larvae with the
122 rationale that resistance results from the recognition of
123 multiple antigens (Ellis et al., 2014). This test is deliberately
124 targeted to the predominant nematode infection in the UK
125 and other temperate climates. Breeders and veterinarians
126 have readily adopted this test and results are reported as
127 an Estimated Breeding Value (EBV) for inclusion in the
128 selective breeding programmes of the Lleyn Performance
129 Recording group.

130

131 2. Material & Methods

132 2.1 *Animals and sample collection*

133 Eighteen farms owned by Performance Recorded Lleyn
134 Breed Society members throughout the UK were involved
135 in the study and bred all the Lleyn animals that were tested
136 (n=5201). Sampling was undertaken between the months
137 of July-September to coincide with exposure to
138 *Teladorsagia circumcincta* through grazing. For lambs
139 sampled between 2014 and 2017 the average age at
140 sampling was 193days of age. The age distribution of
141 animals in the study is shown in Figure 1.

142

143 Saliva samples were collected by insertion of a dental swab
144 (Robinson Healthcare, 12mm), secured by forceps, into the
145 cheek pouch of the animal. Swabs were sealed in 15ml
146 falcon tubes and saliva recovered by centrifugation at 449
147 rcf for 5 minutes. Saliva was then frozen at -20°C before use
148 in ELISA.

149

150 2.2 Antigen and ELISA

151 *T. circumcincta* antigen was prepared by homogenisation of
152 1 million L3 in 10mM Tris-HCL with addition of protease
153 inhibitors (0.5M EDTA, 0.5M EGTA, 1M NEM, 0.33M PMSF,
154 0.1M TPCK, 1mM pepstatin) and 2% sodium deoxycholate.
155 Homogenisation was performed at 30Hz for 6 mins in a
156 Retsch MM400 mixer mill. Antigen was passed through a
157 0.2µm syringe filter and frozen before use in ELISA.

158
159 Antigen-specific salivary IgA ELISA: 96 well BD Falcon
160 ELISA plates were coated with 5µg/ml L3 antigen diluted in
161 0.06M carbonate buffer (0.04 M NaHCO₃, 0.02 M NaCO₃,
162 pH9.6) and incubated overnight at 4°C. Non-specific
163 binding was blocked with 4% Marvel milk powder in
164 Phosphate-buffered saline with 0.1% Tween 20 (PBST) for
165 2 h at 37 °C. Wells were washed three times in PBST after
166 each subsequent step. Saliva samples were added at 1:4
167 dilution using PBST as a diluent and incubated for 30
168 minutes at 37 °C. Isotype-specific detection antibody
169 horseradish peroxidase conjugated rabbit anti-ovine IgA
170 (AbD serotec AHP949P) was added at 1:15 000. Plates were
171 incubated for 30 minutes at 37 °C. An additional wash in
172 distilled water was carried out before developing with 3,3',
173 5,5'-tetramethylbenzidine (TMB) peroxide substrate
174 (Pierce TM), at room temperature for 5 min. Optical density
175 (O.D.) was read at 450nm using a spectrophotometer.
176 Results were expressed as an O.D. Index calculated as
177 (sample O.D. – control O.D.)/ (high-responder O.D. – control
178 O.D.). This O.D. Index and animal pedigree were used to
179 generate an estimated breeding value for antigen-specific
180 IgA (IgA EBV).
181

182 To ensure standardisation of IgA responses from year to
183 year the high responder pool was created from 6-10 sheep
184 and this provides enough material to test thousands of
185 animals. When a new batch of high responder pool is
186 created the reactivity of the previous pool and the new pool
187 are carefully compared by testing multiple samples with
188 both batches.

189

190 *2.3 Calculation of IgA Estimated Breeding Value (EBV)*

191 At the time of producing IgA EBVs, raw antigen-specific IgA
192 data were available for 5,201 Lleyrn animals, measured
193 between 2014 and 2016 from 15 flocks. Basic data edits
194 were undertaken to remove data for animals that were
195 sampled multiple times or outside of 100 to 350 days of
196 age. To normalise the IgA phenotype distribution a Box-Cox
197 procedure was applied using a square root transformation
198 and extreme outliers removed. To allow genetic parameter
199 estimation with the other traits routinely evaluated in the
200 national Lleyrn genetic evaluation, Faecal Egg Count (FEC)
201 (log transformed), birth weight (kg), 8 week weight (kg), 21
202 week weight (kg), ultrasound muscle depth (mm),
203 ultrasound fat depth (mm) and ewe weight at tupping (kg)
204 were extracted from the genetic evaluation database for the
205 flocks that recorded antigen-specific IgA. For genetic
206 parameter estimation, animals that were fostered, the

207 result of embryo transfer, not purebred or from a litter size
208 greater than 3 were removed from the dataset. Any record
209 which was part of a small (< 5 animals) or single sire
210 contemporary group were set to missing, as were records
211 outside 3 standard deviations from the contemporary
212 group mean.

213

214 Contemporary groups (CG) were as defined in the routine
215 national genetic evaluations (Ceyhan, 2015). For birth- and
216 8 week- weight the CG was defined as flock of birth, season
217 and sex (Flock-Season-Sex CG). The same definition, but
218 with the inclusion of a user defined management group
219 (Flock-Season-Sex-Managementgroup CG) was used for 21
220 week weight, ultrasound muscle and fat depth and Faecal
221 Egg Count. In all cases, season was based on date of birth
222 and within year (defined from August to July) was sliced so
223 that consecutive animals were not more than 28 days apart
224 in age and the total time span of a CG was not more than
225 155 days (5.5 months). The CG for ewe weight at tugging
226 was the herd and year of tugging (Tugging herd-year CG).
227 For IgA it was decided to use a similar CG as for 21 week
228 weight, ultrasound scans and FEC, however including the
229 IgA management group. A 5 generation pedigree was built
230 for animals with phenotype information.

231

232 For both parameter estimation and the calculation of EBVs
233 the statistical models used for routine genetic evaluation
234 were used for all traits other than IgA. The statistical
235 models fitted were as follows;

236

237 Birth weight = Flock-Season-Sex CG + litter size born + dam
238 age + animal genetic effect + dam genetic effect +
239 permanent environment effect

240 8 week weight = Flock-Season-Sex CG + litter size reared +
241 dam age + animal genetic effect + dam genetic effect +
242 permanent environment effect

243 21 week weight / ultrasound muscle depth /ultrasound fat
244 depth / FEC = Flock-Season-Sex-Managementgroup CG +
245 litter size reared + dam age + scanning age + Flock-Season-
246 Sex-Managementgroup CG * scanning age + animal genetic
247 effect

248 Ewe weight at tugging = Tugging herd-year CG + animal
249 genetic effect

250

251 The CG, litter size and dam age terms were fitted as fixed
252 class effects. CG was as defined above, litter size born was
253 the number of lambs born in the same litter, litter size
254 reared was the number of lambs born and raised in the
255 same litter. Dam age was the age of the dam in years, with
256 dams over 5 years of age being recorded as 5 yrs.

257

258 Scanning age was the age of the animal at the time of the 21

259 week weight and ultrasound scans were measured. It is

260 recorded in days and was fitted in the model as a fixed

261 covariate.

262

263 Fitted as random effects were the additive, dam and

264 permanent environment genetic effects.

265

266 The final statistical model for IgA was as follows;

267
$$\text{IgA} = \text{Flock-Season-Sex-ManagementGroup CG} + \text{litter size}$$

268
$$\text{born} + \text{age when IgA sample taken} + \text{animal genetic effect}$$

269

270 Model terms were fitted in the same manner as above, with

271 age at IgA sample being recorded in days and fitted as a

272 fixed covariate.

273

274 ASReml (Gilmour, 2009) was used to estimate genetic

275 parameters to produce EBVs. To estimate (co)-variance

276 components, a series of uni- and bi- variate animal models

277 (described above) were fitted. The software MiX99

278 (Lidauer, 2011) was used to produce EBVs based on the

279 variance components estimated and the models described

280 above.

281

282 3. Results

283 After basic data edits antigen-specific IgA records were
284 available for 5,188 animals, of which 4,473 also had FEC
285 records available in the national Lleyn genetic evaluation.
286 Table 1 describes the raw data that was available for
287 genetic parameter estimation. A small to moderate
288 heritability of 0.16 (0.04) was estimated for antigen-specific
289 IgA transformed to a normal scale (Table 2), this was
290 double that of the heritability calculated for FEC in the same
291 cohort (Table 2). In addition to being heritable, there was
292 sufficient genetic variation to enable selection of animals
293 for increased antigen-specific IgA activity.

294

295 Genetic relationships estimated from bi-variate analysis are
296 reported in Table 2. Moderate genetic correlations
297 between IgA and FEC were estimated with a negative
298 correlation (-0.26 (0.02)) between IgA and FEC Strongyles.
299 These correlations indicate that as IgA increases
300 genetically, FEC Strongyles decreases improving host
301 resistance for these types of worms. A positive correlation
302 (0.27 (0.19)) between IgA and FEC Nematodirus was also
303 observed, however this correlation estimate was not
304 significantly different from 0.

305

Parameters were unable to be estimated for birth and eight week weight due to convergence issues. The correlations between antigen-specific IgA activity and production traits were not significantly different from 0. These results indicate that selection for antigen-specific IgA is possible without any adverse influence on production traits for the Lley breed.

Based on the genetic parameters estimate, EBVs were produced for IgA with the only correlation fitted for IgA being with FEC Strongyles (-0.26 (0.02)). The average IgA EBV was 0.00 and ranged between -0.17 and 0.18 with a standard deviation of 0.01. A simple correlation between sire EBV and progeny IgA was undertaken for sires with more than 10 progeny recorded for IgA. After adjusting the phenotypes for the fixed effects fitted in the statistical model, a correlation of 0.91 was observed (Figure 2). This relationship suggests that sires with higher EBVs for IgA will produce offspring with genetics to produce higher IgA responses.

4. Discussion

This study has shown that antigen-specific salivary IgA responses are heritable and negatively correlated with FEC making them suitable to identify animals resistant to

331 nematodes for use in selective breeding programs. Antigen-
332 specific IgA responses have twice the heritability of FEC
333 (which is currently used to identify resistant animals)
334 suggesting that selection based on IgA would be more
335 efficient. Furthermore we predict there would be no
336 adverse effect of selection on production traits
337 (correlations between antigen-specific IgA and fat and
338 muscle deposition were not significantly different from
339 zero). This is in contrast to ecological theory which
340 predicts that a trade-off between immunity and host-
341 growth will exist due to competing energetic needs
342 (Klasing, 2004). It is possible that this trade-off is not
343 evident for the measures of immunity and growth used
344 here because the IgA response to *T. circumcincta* is not
345 known to cause pathology and a relative protein deficiency
346 as is the case for the IgE response (Stear et al., 2003).

347

348 Previous research has focussed on plasma IgA responses to
349 *T. circumcincta* L4 antigens (JP et al., 2014; Strain et al.,
350 2002). Here we opted to use L3 antigens as they are easier
351 and less costly to produce than L4 sourced antigens. There
352 is good evidence that L3 antigens can be used as a proxy for
353 L4 (McRae et al., 2014) and a strong correlation between
354 plasma IgA responses to L3 and L4 antigens has previously
355 been shown (Stear et al., 1995). Despite being easier to

356 produce it is likely that responses to L3 antigens will have
357 weaker relationships to FEC and perhaps productivity than
358 L4 and in the future the use of L4 antigens or a suite of L4
359 recombinant antigens in ELISA may be advantageous.
360 Indeed the heritability of IgA activity to L4 antigens in
361 plasma of Scottish Blackface sheep (0.56 ± 0.11) (Strain et
362 al., 2002) was higher than that measured here to L3
363 antigens in saliva of Lleyen sheep. It should be noted
364 however that these two heritabilities are not directly
365 comparable as different data transformations and statistical
366 models were used. Importantly in each case the heritability
367 of IgA responses was approximately twice that of FEC
368 (Bishop, 1996).

369

370 The strength of the relationship between salivary IgA and
371 plasma IgA or indeed mucosal IgA at the site of infection
372 (abomasum) remains to be determined. However, the
373 majority of IgA present in saliva is derived from B-
374 lymphocytes that migrate from the gut associated lymphoid
375 tissue (Brandtzaeg, 2007a, b). This underlying biology and
376 previous use of L3 antigens in place of L4 (McRae et al.,
377 2014) supports our use of salivary IgA responses to L3
378 antigens as an indicator of the protective mucosal IgA
379 response to L4 antigens. This rationale was upheld by the
380 favourable genetic correlation with FEC that we observed.

381

382 We advocate using salivary IgA as an indicator of resistance
383 to *T. circumcincta* as on practical level saliva is easy to
384 collect for farmers or veterinarians and there is no
385 possibility of animals not providing a sample. Samples are
386 also easy to process in large numbers and, as salivary IgA is
387 quite stable, can be shipped at room temperature.
388 Alternatively the saliva (or saliva soaked swab) can be
389 stored at -20°C prior to analysis without impacting the
390 result.

391

392 Selection for resistance to nematodes based on FEC in the
393 UK requires an average flock level of 100 eggs per gram
394 (epg) or higher (Signet Breeding Services, 2014). For FEC it
395 is also crucial that lambs have not been treated with
396 anthelmintics for at least 4 weeks. IgA activity is less
397 affected by anthelmintic use and (as with FEC) flock EBV
398 will be useful for animals that have been exposed to similar
399 levels of infection by grazing the same pasture. Currently
400 the Lleyn breeders are utilising both FEC and IgA EBVs, as
401 the required flock responses in IgA activity are still to be
402 determined.

403

404 In this study we measured antigen-specific responses to *T.*
405 *circumcincta* larval antigens. Of course the animals were

406 grazed on pasture and so would be exposed to and infected
407 with other parasitic nematodes (e.g. *Nematodirus battus*,
408 *Cooperia curticei*, *Oesophagostumum* and *Trichostrongylus*
409 species). We would anticipate some cross-reactivity in the
410 antibody response to *T. circumcincta* and these other
411 species and this may be beneficial to the host. However,
412 larval culture for speciation performed on a subset of faecal
413 samples revealed these lambs to be predominantly infected
414 with *T. circumcincta* (unpublished). In addition to the
415 favourable negative correlation between IgA and FEC
416 Strongyles, a positive correlation between IgA and FEC
417 *Nematodirus* was observed (Table 2). However, this
418 correlation estimate was not significantly different from 0.
419 The difference in direction was unexpected, given that a
420 strong positive genetic correlation (0.61 (0.16)) between
421 the two FEC traits was estimated. It is worth noting that
422 *Nematodirus* counts are zero-inflated (Denwood et al.,
423 2008) and so estimates using faecal egg counts for this
424 species need to be treated cautiously.

425

426 5. Conclusions

427 Salivary IgA responses to *T. circumcincta* larval antigens
428 can be used to identify animals resistant to nematodes for
429 inclusion in selective breeding programs. Antigen-specific
430 IgA responses have twice the heritability of FEC (which is

431 currently used to identify resistant animals) suggesting that
432 selection based on IgA would be more efficient. On a
433 practical level saliva is easy to collect for farmers or
434 veterinarians and there is no possibility of animals not
435 providing a sample. The research reported here was
436 conducted on Lleyn sheep, however the IgA test is
437 appropriate for use throughout the sheep breeding industry
438 as a tool for breeders to identify animals resistant to
439 nematode infection. Selection of animals based on the IgA
440 test could play an important role in the integrated
441 management of *T. circumcincta* and reduce the need for
442 treatment with anthelmintics.

443

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459

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567 Table 1: A summary of the edited Lleyn data used to

568 estimate genetic parameters

Trait	n	average	min	max	Std. dev
birth weight (kg)	66,626	4.14	2.0	7.0	0.96
8 week weight (kg)	268,594	19.17	5.7	32.8	4.36
21 week weight (kg)	102,035	35.76	13.0	58.6	7.44
Ultrasound muscle depth (mm)	63,499	24.08	13.7	34.5	3.41
Ultrasound fat depth (mm)	62,825	2.60	0.1	7.13	1.36
ewe weight at tupping (kg)	13,398	54.71	20	90	12.12
Faecal egg count – Strongyles (log transformed)	5,840	5.12	0.0001	9.00	2.08
Faecal egg count – Nematodirus (log transformed)	5,840	1.89	0.0001	7.62	2.36
transformed IgA	4,622	0.71	0.03	1.99	0.35

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571 Table 2: Estimates of phenotypic variances, heritability and
 572 phenotypic and genetic correlations between transformed
 573 IgA and production traits of UK Lleyn sheep

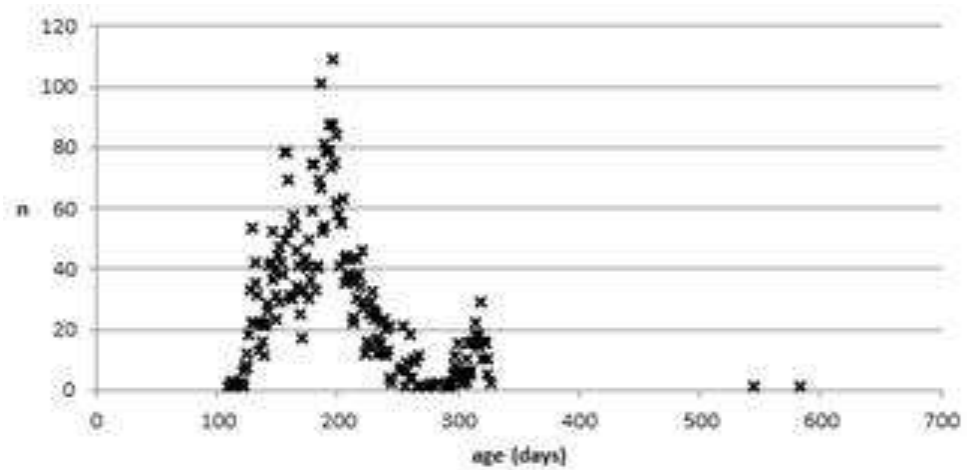
Trait	Phenotypic Variance	Heritability	Phenotypic correlation with IgA	Genetic correlation with IgA
transformed IgA	0.08 (0.002)	0.16 (0.04)	-	-
Faecal egg count – Strongyles (log transformed)	2.68 (0.06)	0.08 (0.03)	-0.002 (0.02)	-0.26 (0.02)
Faecal egg count – Nematodirus (log transformed)	4.55 (0.11)	0.15 (0.04)	0.007 (0.02)	0.27 (0.19)
Ultrasound muscle depth (mm)	4.85 (0.09)	0.37 (0.03)	0.05 (0.02)	0.08 (0.12)
Ultrasound fat depth (mm)	1.04 (0.02)	0.41 (0.03)	0.04 (0.02)	0.04 (0.12)
21 week weight (kg)	20.54 (0.25)	0.45 (0.02)	0.02 (0.02)	-0.02 (0.11)
ewe weight at tupping (kg)	37.81 (0.85)	0.37 (0.03)	0.04 (0.04)	0.25 (0.15)

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576 Figure 1: Age distribution of animals sampled between 2014
577 and 2017. Animals greater than 350 days old were excluded
578 from final analysis. The mean age at sampling for all animals
579 was 193 days. For males the mean age was 182 days and for
580 females 196.

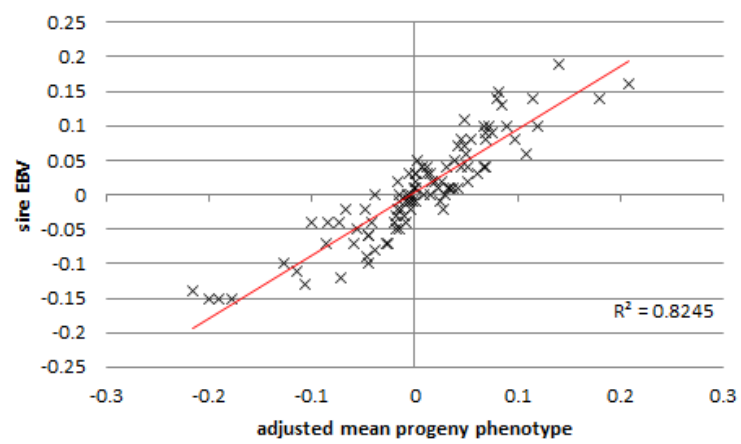
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584 Figure 2: Sire IgA EBV and the mean progeny IgA phenotype
585 adjusted for fixed effects



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